Microbiol. Biotechnol. Lett. (2022), 50(1), 110–121
http://dx.doi.org/10.48022/mbl.2201.01003
pISSN 1598-642X eISSN 2234-7305

MBL
Microbiology and Biotechnology Letters

A Microbial Consortium for the Bioremediation of Sulfate-Rich Wastewater Originating from an Edible Oil Industry

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Received: January 12, 2022 / Revised: February 3, 2022 / Accepted: February 4, 2022

The effluents from industries processing vegetable oils are extremely rich in sulfates, often exceeding the maximum concentration allowed to release them to the environment. Biological sulfate reduction is a promising alternative for the removal of sulfates in this type of wastewater, which has other particularities such as an acidic pH. The ability to reduce sulfates has been widely described for a particular bacterial group (SRB: sulfate-reducing bacteria), although the reports describing its application for the treatment of sulfate-rich industrial wastewaters are scarce. In this work, we describe the use of a natural SRB-based consortium able to remove above 30% of sulfates in the wastewater from one of the largest edible oil industries in Peru. Metataxonomic analysis was used to analyse the interdependencies established between SRB and the native microbiota present in the wastewater samples, and the performance of the consortium was quantified for different sulfate concentrations in laboratory-scale reactors. Our results pave the way towards the use of this consortium as a low-cost, sustainable alternative for the treatment of larger volumes of wastewater coming from this type of industries.

Keywords: **Sulfate-reducing bacteria, wastewater, biological treatment, microbial consortia, metataxonomics**

Introduction

Vegetable oils are a rich source of nutrients and one of the main foods or food ingredients consumed worldwide. In the 2019/2020 crop year, over 180 million metric tons of edible vegetable oils were produced [1]. Soap stock is a by-product of crude vegetable oil refining which contains soaps (sodium salts of fatty acids), neutral oils and different impurities such as phosphatides. Fatty matter of the soap stock typically ranges from 15% to 50% and, hence, an extensive amount of fats can still be recovered. To reach that goal, the soap stock is first saponified by adding sodium hydroxide to a pH of up to 11 at high pressure (10 bar) and high temperature (> 85℃) (Fig. S1). Next, a strong acid is added, namely sulfuric acid, until the pH lowers down to 2–3 (Fig. S1). The purpose of this step is to recover the fatty acids through the soap splitting reaction. Consequently, the by-product of this process is an acidic lipid-rich wastewater characterized by a high sulfate concentration, recurrently reaching concentrations of up to > 30,000 ppm. This wastewater must be treated to lower such amounts of sulfates before it can be discharged.

The maximum concentration of sulfates that wastewaters may contain is legislated specifically by each country. In the specific case of Peru, surface water

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intended for human consumption must not contain over 250–500 ppm of sulfates in suspension (El Peruano, Decreto Supremo N° 004-2017-MINAM), while in wastewater the limit is 1,000 ppm. Sulfates are water-soluble inorganic ions, albeit there are some exceptions such as $CaSO_4$. They are chemically inert, non-volatile, and non-bioaccumulative molecules, and in water are not toxic unless at very high concentrations [2, 3]. The consumption of water with a concentration of sulfates higher than 1,000 ppm commonly results in laxative effects, in addition of a noticeable taste [4]. Current restrictions and legislations on sulfate release in the environment mainly aim to reduce the salt content of surface waters and/or to minimize acid in sewers [5, 6]. However, high sulfate concentrations can unbalance the natural sulfur cycle [7].

Acidic lipid-rich wastewater from the soap stock splitting process must be dealt with to decrease the sulfate concentration prior to its release into the environment and, to date, various chemical treatments have been developed to deal with high sulfate concentrations, including chemical treatment with mineral precipitation, membrane technologies, and ion exchange technologies [8, 9]. A cheaper alternative is the use of a biological treatment which can handle larger volumes of wastewater [10, 11]. The biological removal of sulfates can be carried out by sulfate-reducing bacteria (SRB), a taxonomically and metabolically diverse group of microorganisms able to carry out an anaerobic respiration using sulfate, thiosulfate or sulfite as a terminal electron acceptor and an organic substrate or hydrogen as an electron donor [12].

The genus *Desulfovibrio* (phylum *Desulfobacterota*, formerly class *Deltaproteobacteria*), is one of the most widely used SRB used for the sulfate bioremediation processes [12–16]. For SRB to carry out sulfate reduction in wastewaters, they need to cooperate with other microorganisms which provide them with suitable electron donors from complex nutrients through hydrolytic, acidogenic and acetogenic processes (Fig. 1) [17].

The present work aimed to develop a fixed bed bioreactor optimised for the bioremediation of sulfates in acid water originating from an edible oil refining facility. To the best of our knowledge, this is the first bioreactor specifically conceived to bioremediate sulfates from acidic lipid-rich wastewaters.

Materials and Methods

Acid wastewater to be treated

The acidic wastewater samples used in this study originated from an edible oil industry in Peru, after the

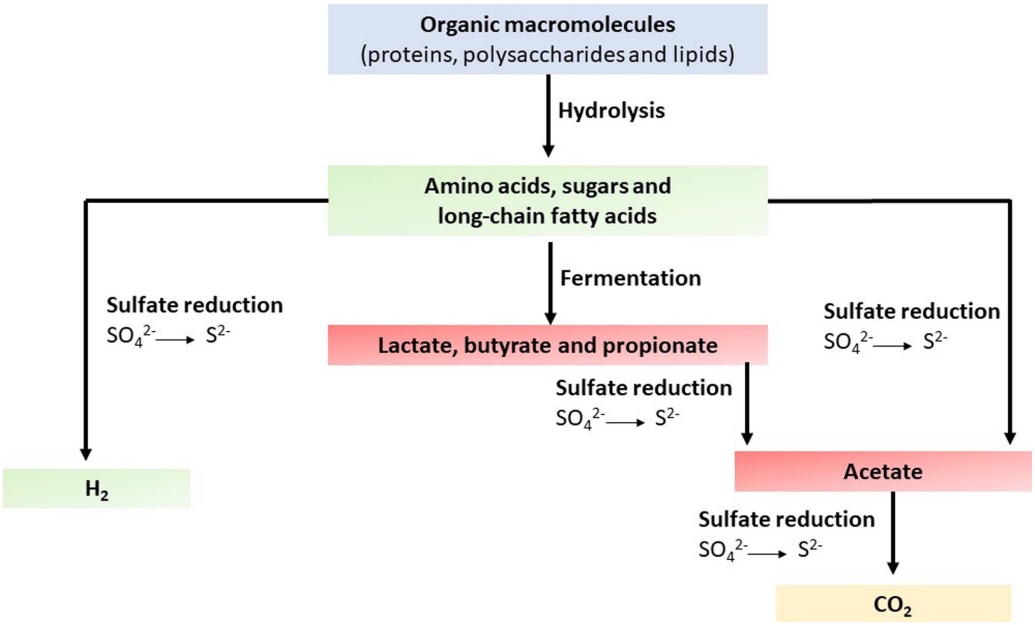

Fig. 1. Sequential pattern of metabolic pathways which take place during the sulfate bioremediation process of wastewater coming from soapstock splitting.

Table 1. Main physicochemical parameters of the acidic lipid-rich wastewater collected after the soap stock splitting process.

Parameter	Value
Acidity	8,733.5 mg$CaCO_3$/L
pH	2.51
Conductivity	47,400.00 µS/cm
Total suspended solids	167 mg/l
Settleable solids	< 1.0 ml/l/h
NH_4^+	8.950 mg/l
Sulfide	< 0.0010 mg/l
Biochemical oxygen demand (BOD)	196.3 mg/l
Chemical oxygen demand (COD)	21,392.1 mg/l
Total organic carbon (TOC)	3,678.7 mg/l
Oils and fats	16 mg/l
NO_3^-	4.781 mg/l
NO_2^-	155.155 mg/l
PO_4^{3-}	8,479.54 mg/l
SO_4^{-2}	25,700.02 mg/l

soap stock splitting process of sunflower and soya oils. The physicochemical properties of the acidic wastewater are listed in Table 1. The wastewater had a pH of 2.5 and a high conductivity (47,400.00 µS/cm). Most of the solids were not settleable, suggesting a hydrophobic nature of those solids. The wastewater was rich in organic matter, including oils and fats, as evidenced by the high biological oxygen demand, chemical oxygen demand, and total organic carbon values. The higher COD value in comparison to BOD and TOC values suggests that wastewater had a high concentration of organic matter which was prone to be oxidated but a priori not biologically. In addition of free and esterified fatty acids, the wastewater originating from the soap splitting process could also contains organic phosphates, mono-, di- and triglycerides, sterols, and polyols. These other organic compounds might account for the high DOC (and low BOD) value observed. The exceedingly high concentrations of phosphates (8,479 ppm) and sulfates (25,700 ppm) were noteworthy (Table 1). The ratio COD/SO_4^{-2} of the wastewater sample was 0.83.

The physicochemical parameters of the acidic wastewater used to carry out the research were characterized by The National Institute of Quality (INACAL) of Peru (https://www.gob.pe/inacal) using standardized methods.

Design of the microbial consortium

Two sequential steps were performed to develop a microbial consortium able to carry out sulfate bioremediation in the wastewater to be treated in the present project. The wastewater characterised physiochemically was the same as the one used for subsequent experiments. First, a natural microbial consortium capable of carrying out the hydrolysis of macronutrients and the subsequent fermentation of monomers was obtained from an acidic wastewater sample from an edible oil refining facility. Then, the resulting consortium was artificially supplemented with SRBs capable of carrying out sulfate reduction.

To obtain the natural consortium, 100 ml of the acid wastewater was first neutralized up to pH 7.0 with NaOH. Then, the neutralised wastewater was transferred to a 1 L Pyrex beaker and incubated at 30℃ for 72 h to be naturally contaminated by environmental microorganisms. After verifying with an optical microscope that the neutralised water was colonised by microorganisms, 100 ml were transferred to a sterile Pyrex bottle (1 L) and further incubated without shaking for 15 days.

In parallel, SRB were isolated from a red-pigmented sample of a laboratory scale anaerobic digester operating in Jena (Germany). Technical details of the anaerobic digester and the microbial community structure inhabiting the digestor were previously described [18]. SRB were enriched, isolated, and subsequently cultivated using the Postgate medium (=DSM medium 63; https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium63.pdf). The medium was made anoxic by boiling and subsequent cooling under an N_2 atmosphere. Upon cooling, the gas phase was changed to N_2/CO_2 (90/10, v/v) and aliquots of 9 ml medium were anaerobically dispensed into Hungate anaerobic tubes under a N_2/CO_2 (90/10, v/v) atmosphere. Incubations were carried out in the dark at 30℃. After 2 months, two strains (DWN_Desulf_01 and DWN_Desulf_02) were isolated in pure culture by repeated use of the roll tube technique [19]. The purity of the culture was checked by optical microscopy (Fig. 2) and by aerobic and anaerobic growth tests in complex medium containing yeast extract (2 g/l) and glucose (5 mM). The taxonomic identity of both strains was established by sequencing of the almost full 16S rRNA gene using universal primers and

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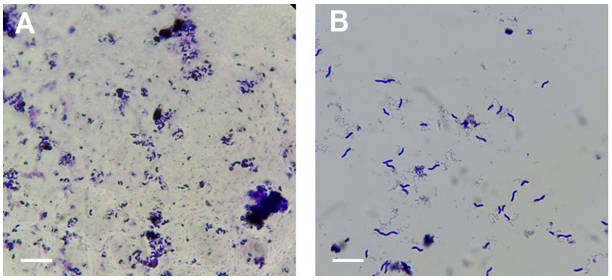

Fig. 2. Cell morphology of strains DWN_Desulf_01 (A) and DWN_Desulf_02 (B) under the optical microscope at 64× magnification and stained with crystal violet glass. Size bars, 5 μm.

following the methodology previously described [20]. Taxonomic assignment of the two strains was done using the EzBiocloud online database (https://www.ezbiocloud.net/), and a phylogenetic tree comprising type strains and environmental clones was constructed using the methodology previously described by Pascual *et al*. [21]. The phylogenetic tree was obtained using the Neighbour-Joining method and the K2P evolutionary model (Fig. S2).

The two *Desulfovibrio* strains were incorporated into the microbial community that had naturally colonised the wastewater. Each SRB strain was inoculated at a final concentration of $5 \times 10^{+3}$ genomic equivalents/ml. Finally, the microbial consortium underwent a process of adaptive evolution in the laboratory. Briefly, the consortium was incubated at 30℃ for three months, replacing every 15 days one third of the culture volume with new neutralised wastewater.

Design of a fixed bed bioreactor and proof of concept

The sulfate reduction experiments were carried out in 1 L borosilicate glass bottles (bioreactors) (Fig. 3). Each bioreactor contained 50 ml of lightweight expanded clay aggregate (LECA) as solid matrix (Table S1) and 200 ml of wastewater to be treated. The fixed bed bioreactor operated in a batch mode, replacing 50 ml of the total volume of wastewater (200 ml) per week (Table S1). After each batch, once per week, the treated wastewater with a reduced sulfate concentration was discarded. Bioreactors were operated at 25℃ and without agitation to enable low oxygen levels. Since the designed microbial consortium grows optimally at neutrophilic pH, the sulfate-rich acid wastewater to be treated must be neu-

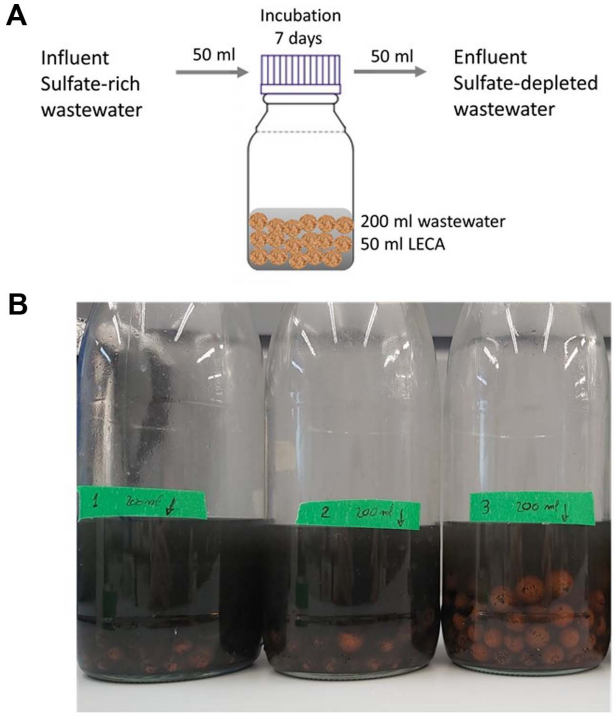

Fig. 3. (A) Graphical representation of the bioreactor indicating the operating parameters, (B) Images of the three bioreactors where the optimal amount of sulfates to be treated per batch of 7 days was quantified: 25,700 ppm (Reactor 1), 14,400 ppm (Reactor 2) and 8,370 ppm (Reactor 3).

tralised with NaOH (10 N) before use.

To evaluate the ability of the microbial consortium to reduce the sulfate concentration in wastewater, three wastewater samples with different SO_4^{-2} concentration (Reactor 1, 25,700 ppm; Reactor 2, 14,400 ppm; Reactor 3, 8,370 ppm) but identical COD/SO_4^{-2} ratio (0.81) were tested for 35 days. We tested these three initial sulphate concentrations because they covered the range of sulphate concentrations that are usually observed in the acidic lipid-rich wastewater originating from soap splitting reactions. The sulfate concentration of the effluent wastewater from each batch (weekly) was quantified. The quantification of sulfates in wastewater samples was carried out by the company Laboratorios Tecnológicos de Levante (Paterna, Spain; https://www.ltlevante.com/) with a turbidimetric method according to the standard method SM 4500-SO4 A [22].

To compare the sulfate reduction values among samples, the statistics one-way ANOVA and Tukey test

for multiple comparisons were applied. All statistical analyses have been performed with the Rcmdr package (version 2.7-1) embedded in the R tool [23].

Taxonomic analysis of the microbial consortium

Taxonomic composition of the microbial consortium colonizing the bioreactors was studied by next-generation sequencing (NGS) of 16S rRNA amplicons. Briefly, metagenomic DNA was extracted using the Power Soil DNA isolation kit (MO BIO Laboratories, USA), and the resulting DNA was quantified using the QUBIT dsDNA HS-high sensitivity kit (Invitrogen, USA). The conserved regions V3 and V4 (459 bp) of the 16S rRNA gene were then amplified using forward and reverse primers: 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′ and 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3′, respectively [24]. Amplification was carried out using the KAPA HiFi HotStart ReadyMix PCR kit (KK2602) and the following PCR cycle: initial denaturation at 95℃ for 3 min; 25 cycles of amplification (30 s at 95℃, 30 s at 55℃, 30 s at 72℃); and 5 min of extension at 72℃. Amplicons were mixed with Illumina sequencing adaptors and dual-index barcodes (Nextera XT index kit v2, FC-131-2001). Libraries were normalized and merged before the sequencing. Then, the pool containing indexed amplicons was loaded onto the MiSeq reagent cartridge v3 (MS-102-3003), spiked with 10% PhiX control to enhance the quality of the sequencing. Finally, paired-end sequencing (2 × 300 bp) was carried out on the Illumina MiSeq sequencing system. Illumina outcomes were analysed via Qiime2 software [25]. The Demux plugin was used to assess the quality of reads, and the Qiime2-integrated Dada2 pipeline was employed for trimming and joining the sequences, removing chimeras, and detecting sequence variants (>99.9% of similarity). The classify-Sklearn module (feature-classifier plugin) was applied for assessing the taxonomy of each sequence variant, using the SILVA (v. 138) database as reference [26]. Taxonomic composition of evolved consortia was visualized as Krona charts [27].

The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequences of strains DWN_Desulf_01 and DWN_Desulf_02 are OM403593 and OM403594, respectively. The GenBank Short Read Archive (SRA) sequence accession numbers for 16S rRNA gene amplicon. The 16S rRNA gene amplicon data set was deposited in GenBank under the SRA accession number SRR17854623 (BioProject number PRJNA801085).

Functional characterization of the microbial consortium

PICRUSt2 (phylogenetic investigation of communities by reconstruction of unobserved states, version 2) was performed, based on metataxonomic data, to predict the functional aspects of the microbial community of the bioreactors [28, 29].

Quantification of *Desulfovibrio* cells via qPCR

The number of strains belonging to the genus *Desulfovibrio* in the wastewater samples was quantified by real-time PCR (qPCR) using the genus-specific primers Dsv 691F (5'-CCGTAGATATCTGGAGGAACATCAG-3') and Dsv 826R (5'-ACATCTAGCATCCATCGTTTACAGC-3') [30]. Experiments were performed using an Applied Biosystems 7500 Fast Real-Time PCR System, and each reaction was set up in duplicate in a volume of 20 µl in 96-well optical-grade PCR plates, sealed with optical sealing tape (Applied Biosystems). Amplification reactions were detected with Power SYBR® Green Master Mix (Applied Biosystems) mixed with the forward and reverse primers (0.3 µM each) and 2.5 µl of DNA. The following temperature profiles were used for amplification: one cycle at 95℃ for 10 min, 40 cycles of denaturation at 95℃ (15 s), followed by 60℃ (60 s). Melt curve analyses were done by slowly heating the PCR mixtures from 60 to 95℃ (1℃ per cycle of 15 s), as end point assays to confirm PCR specificity. Standard curves were calculated for quantification purposes using 10-fold dilutions of DNA extracted from strains DWN_Desulf_01 and DWN_Desulf_02 covering the range from 2.5 to 2.5×10^5 genome equivalents per reaction, which were calculated assuming that 1 ng of DNA equals 2.5×10^5 times the entire genome of *Desulfovibrio* strain.

Results and Discussion

Taxonomic and functional structure of the microbial consortium

The acidic wastewater originated after the soap stock splitting process is a serious environmental problem due to its high concentration of sulfates. In the present

study, a simplified microbial consortium has been developed to bioremediate this type of wastewater before discharging it into the sewerage system. The consortium was obtained by adding two SRB recovered from a red-pigmented sample of a laboratory-scale anaerobic digester into a simplified microbial consortium evolved from an acidic wastewater sample naturally colonized. Members of the genus *Desulfovibrio* were selected as appropriate bacteria to carry out sulfate reduction as their usefulness in other bioremediation processes has been previously demonstrated [3, 10, 11].

To taxonomically characterize the microorganisms of the evolved consortium, a metataxonomic study based on 16S rRNA gene amplicons was performed. After the process of filtering, 52,938 high-quality sequences were retained for further studies. The consortium was composed of 138 different bacteria (amplicon sequence variants levels) belonging to the phyla *Proteobacteria*, *Firmicutes*, *Desulfobacteriota* (formerly class *Deltaproteobacteria*), *Verrucomicrobiota*, *Actinobacteriota*, and *Bacteroidota* (Fig. S3). Shannon's diversity index H' was 3.341, and Simpson's dominance index (1-D) was 0.94. These results suggested that the community was characterized by a few numerically dominant bacteria and many numerically minority bacteria. Specifically, the dominant taxa were members of the genera *Pseudomonas* (33.6%), *Enterobacter* (7.9%), *Desulfovibrio* (6.8%), Lachnoclostridium (6.7%), *Sedimentibacter* (6.5%) and *Anaerotruncus* (6.2%) (Fig. 4).

According to the metataxonomic study, the only SRB identified in the consortium were members of the genus *Desulfovibrio* (Fig. 4). Specifically, the consortium harboured $3.41 \pm 1.96 \times 10^{+7}$ genomic equivalents per ml, and according to the melting curves of the qPCR, only two different strains were identified (Fig. 5). These results suggest that both *Desulfovibrio* strains artificially inoculated in the microbial consortium were able to co-exist with other bacteria over time. Based on the 16S rRNA gene sequences, the closest type strains of DWN_Desulf_01 and DWN_Desulf_02 were *Desulfovibrio vulgaris* DSM644(T) (99.8% gene sequence similarity) and *Desulfovibrio gigas* DSM 1382(T) (98.3%), respectively. Furthermore, strain DWN_Desulf_01 was closely related to an uncultured clone inhabiting a gas fed lab-scale reactor (DQ447177), while DWN_Desulf_02 was related to an uncultured clone reported from a sulfidogenic bioreactor (EF055876) suggesting that both strains are adapted to living in environments where sulfate reduction takes place (Supplementary Fig. S2).

Interestingly, methanogenic archaea which could

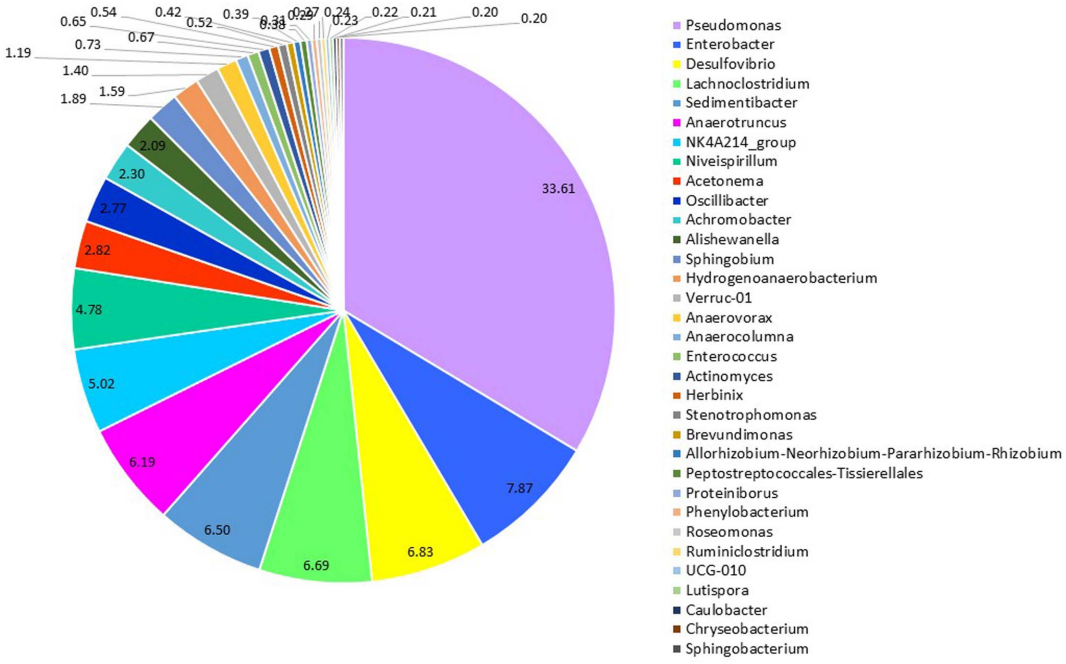

Fig. 4. Pie chart showing the taxonomic composition of the microbial consortium at the genus level.

compete with SRB for electron donors were not identified in the consortium [31–33]. In contrast, homoacetogenic bacteria such as *Acetonema* spp. have been identified in the consortium. The presence of this competitor may be explained by the COD/SO_4^{-2} of the acidic wastewater used in the experimental phase, namely 0.81. According to Van Den Brand *et al.* [33] a maximum COD/SO_4^{-2} ratio of 0.7 is theoretically required to accomplish complete COD removal from wastewater. With an excessive presence of sulfate ($COD/SO_4^{-2} < 0.7$), SRB usually outcompete competitors. However, if sulfate is the limiting component ($COD/SO_4^{-2} > 0.7$), competitors can develop in the system, which is unfavourable for the application of SRB [33].

The diversity of bacteria in the consortium covers all the metabolic functional groups required to carry out sulfate bioremediation while exploiting the organic matter present in the acidic water as a raw material (Fig. 1). The first step of the process is the hydrolysis of polymers and complex molecules to oligomers and monomers. The most abundant and diverse hydrolytic enzymes potentially codified by the microbial consortium were esterases (EC 3.1), glycosidases (EC 3.2), peptidases (EC 3.4), hydrolases acting on carbon-nitrogen bonds (EC 3.5) and hydrolases acting on acid anhydrides (EC 3.6) (Table S2). In particular, lipases (EC 3.1.1.3) and phospholipases (3.1.1.4; EC. 3.1.1.32) were the most significant esterases detected in the consortium. Lipases catalyse the hydrolysis of triglycerides to glycerol and free fatty acids, while phospholipases hydrolyse phospholipids into fatty acids and other lipophilic substances (Fig. 1). These results suggest that the main polymers hydrolysed by members of *Enterobacter*, *Anaerotruncus*, Lachnoclostridium and *Sedimentibacter* could be lipids and phospholipids that cannot be recovered after the soap stock splitting process (Table 1 and Fig. 1). Moreover, the identification of enzymes involved in cellulose and hemicellulose hydrolysis suggested that plant debris could also be metabolized (Table S2). The second functional step is the fermentation of oligomers and monomers producing short-chain fatty acids as end products (SCFAs) (Fig. 1).

Fermenting microorganisms release primary alcohols (EC 1.1.1.1), H_2 (EC 1.17.99.7), acetate (EC 2.8.3.12, EC 2.8.3.9) and/or organic acids, including lactate (EC 1.1.1.27), butyrate (EC 2.8.3.8), formate (EC 2.3.1.54), fumarate (EC 4.2.1.2) and propionate (EC 2.8.3.1). Presumably, the microorganisms involved in the fermentative stage were *Enterobacter*, *Anaerotruncus*, Lachnoclostridium and *Sedimentibacter*. These bacteria could be responsible for converting oligomers and monomers into potential carbon sources used by SRBs.

Finally, SRB oxidize the fermentation-derived products using reduced sulfur compounds - such as sulfate - as an electron acceptor and releasing H_2S as a by-product [12]. Specifically, *Desulfovibrio* can oxidize a wide spectrum of substrates, including organic and inorganic compounds such as H_2, formate, acetate, fatty acids, ethanol, lactate, pyruvate, fumarate, succinate, and malate [34] (Fig. 1).

The ability of the microbial consortium to process the lipidic raw material originated from the soap stock splitting process into substrates assimilable by the SRBs was key to the system being self-sustaining. Otherwise, the need to supply nutrients for SRBs, such as lactate or acetate, would reduce the economic viability of the process. However, the ratio between organic matter and sulfate must be balanced [33]. A nutrient deficiency would result in a decrease in SRB growth and thus in the ability of the system to bioremediate sulfates. In contrast, an excess of nutrients could promote the growth of undesirable microorganisms such as methanogens [33].

Proof of concept of the bioreactor to bioremediate sulfates from lipid-rich wastewater

We tested, at laboratory scale, the designed fixed-bed bioreactor aimed at the bioremediation of sulfate in wastewater originating from the edible oil industries. To the best of our knowledge, this is the first system specifically conceived to bioremediate sulfates from this kind of wastewater. The bioreactor consisted of a one-litre borosilicate glass bottle filled with 50 ml of LECA (Fig. 3). LECA consists of light, expanded clay grains, obtained by expanding clay in rotary kilns at a temperature of about 1200℃. LECA's features include lightweight, low heat conductivity, resistance to fire, chemical durability, and stability. Its lightweight and high specific area (about 525 m^2/m^3) have resulted in the use of this aggregate in various units in water- and wastewater treatment plants [35]. Chemically, these aggregates contain 66% SiO_2, 17% Al_2O_3, 7% Fe_2O_3, and 2.5% CaO, Mg, Ti, Na and K compounds. The main reason for the lightness of LECA grains is the presence of air both

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inside and between the grains. Therefore, we selected this kind of inert material to increase the surface area on which microorganisms could form biofilms and allow the establishment of anaerobic microenvironments for oxygen-sensitive bacteria to grow.

The bioreactor operated in a batch mode containing 200 ml of wastewater, with 1/4 of the volume being replaced weekly. Reactors operating in batch mode have been recurrently used to bioremediate wastewater sulfates [36]. The bioreactor was fed with acidic lipid-rich wastewater obtained after the soap stock splitting process. Due to the neutrophilic nature of the microbial consortium, the wastewater was neutralised before fed the bioreactor. According to Hao [37] SRB prefer an environment around pH 7 and are usually inhibited at pH values below 5.5 or above 9.

The capability of the bioreactor to deal with different sulfate concentrations was assessed as a proof of concept. Specifically, a wastewater originating from an edible oil industry was diluted with sterile distilled water to obtain three different sulfates concentrations, namely 25,700 ppm (Reactor 1), 14,400 ppm (Reactor 2) and 8,370 ppm (Reactor 3). To keep the COD/SO_4^{-2} ratio constant between samples, the wastewater used in reactors 2 and 3 was obtained by diluting the wastewater of the Reactor 1 with distilled water. At first, the three reactors were inoculated with the evolved microbial consortium in 200 ml Postgate medium and incubated for 30 days until the inoculum reached a similar concentration of *Desulfovibrio* spp. ($5.91 \pm 3.58 \times 10^7$) and sulfates (5860 ± 789,9 ppm) (Fig. 5). Subsequently, each reactor was operated for 35 days in a batch mode, by purging 1/4 of the total volume every 7 days. After 35 days, Reactor 1 was the one that showed the best results in terms of sulfate reduction, showing a constant reduction rate over time (30.4 ± 4.2% sulfate reduction) (Figs. 6 and 7). In the case of Reactor 2, the sulfate reduction values were lower during the first 28 days (24.5 ± 8.6% sulfate reduction). Interestingly, it reached a value of 38.62% at day 35. However, no significant differences were found in the percentage of sulfate reduction between reactors 1 and 2 (Tukey test, p-value > 0.05; Fig. 6). Reactor 3 showed the lowest sulfate reduction values (6.27 ± 1.28%), being this result statistically different regarding the other two reactors (Tukey test, p-value < 0.001; Fig. 6). This result could be influenced

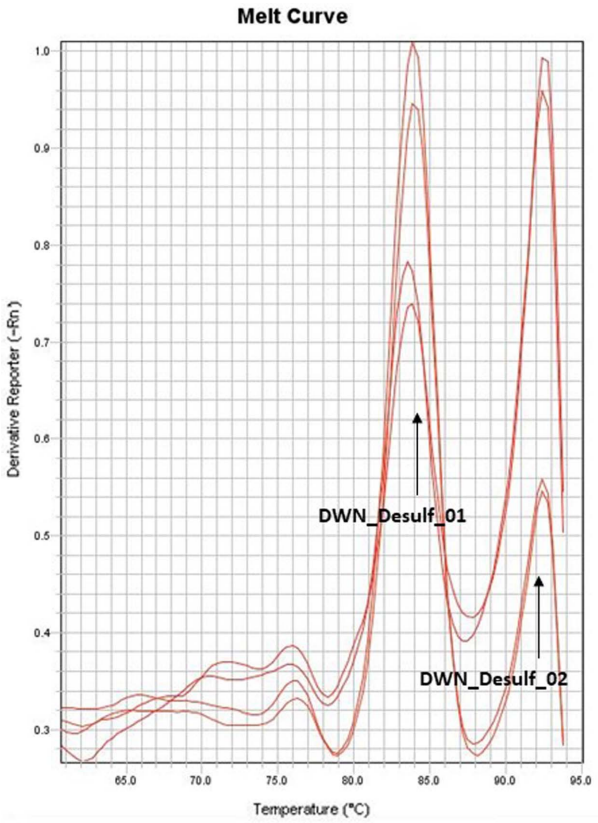

Fig. 5. Melting curve obtained from the microbial consortium using *Desulfovibrio*- specific primers. The analysis was based on four biological replicates. The amplification of two peaks confirms the presence of two *Desulfovibrio* strains with different G+C content.

by the presence of a lower concentration of essential nutrients in the wastewater used in Reactor 3. These results suggest that the microbial consortium is able to bioremediate sulfates in wastewater more efficiently with higher concentrations of nutrients and sulfates, although preliminary tests proved that concentrations close to 40,000 ppm (data not shown) resulted in an inhibition of *Desulfovibrio* spp. growth due to the notable amounts of sulfide species in the medium, produced through sulfate reduction and displaying well-known toxic effects on these bacteria [38]. Sulfate removal values we reached were in line with those observed in other anaerobic bioreactors primed with wastewater, in particular the values ranged between 16 and 68% [39–44]. Several authors have reported sulfate removal rates higher than 80%, however these bioreactors were fed with easily assimilated carbon sources such as

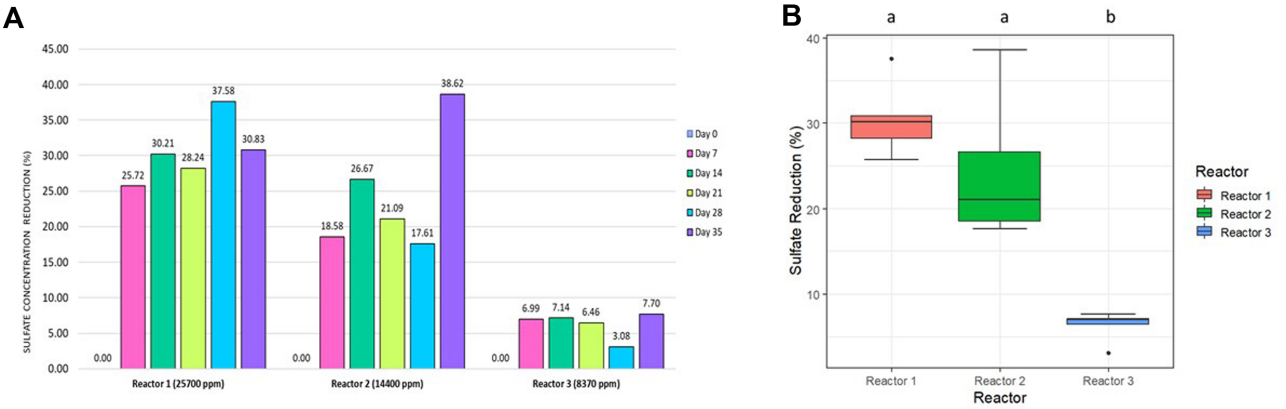

Fig. 6. (A) Sulfate reduction values (%) measured over 35 days in each reactor. Each reactor was supplemented with wastewater with a different sulfate concentration but keeping the ratio COD/SO_4^{-2} constant. (B) Boxplots comparing sulfate reduction values (%) among the three reactors. Different letters at the top indicate significant differences according to the Turkey Contrasts statistic (*p*-value < 0.05).

short-chain organic acids or the raw material had a more optimal CBD/SO_4^{-2} ratio [43, 45, 46]. On the contrary, the aim of our study was to find a compromise between lowering the sulfate concentration to acceptable levels without the need for add easily assimilable nutrients, which would increase the cost of the process.

The main outputs of the bioreactors were different sulfide species, namely S^{2-}, HS^- and H_2S, which co-existed in equilibrium, with the distribution of each species being a function of pH and temperature [47]. For instance, approximately 45% of the total sulfide exists as H_2S_{aq} at pH 7 and 30℃. There is a further equilibrium between aqueous and gaseous H_2S governed by Henry's law, and the escape of H_2S gas depends on the degree of mixing and surrounding ambient H_2S gas concentration [37]. After 35 days, the theoretical concentration of sulfide species was 7812.8 ppm in Reactor 1, 5565.6 ppm in Reactor 2, and 524.8 ppm in Reactor 3. During the operation of the bioreactor, the formation of a black precipitate was observed. Since the acidic wastewater to be treated contains metals such as iron, it is assumed that most of the sulfide produced precipitated in the form of ferrous sulfide.

Sulfide can cause several problems if its concentration increases in the bioreactor. First, it is toxic to acetogenic bacteria and SRB, therefore its accumulation could stop the biological system [59]. However, the retention period of the bioreactors, namely one week, was suitable to reduce sulfates in the wastewater without committing the biological community by the sulfide generated. Literature data with respect to inhibitory concentrations of H_2S or total sulfide vary due to different environmental conditions (e.g. pH, temperature), wastewater characteristics (e.g. presence of iron salts and type of carbon source) and reactor systems (e.g. fixed film) [37]. Previous studies have reported a complete inhibition of *Desulfovibrio* spp. at about 550 mg/l H_2S at pH 6.2 to 6.6 [38].

Second, sulfides have a rotten-egg smell and can cause corrosion problems to pipes, engines, and boilers [47–50]. The sulfides generated during our experiments were discharged into the sewage system after each batch. Although at a laboratory scale the disposal of sulfide in the sewage system is not a problem, this is not the case at the industrial level. The recurrent discard of sulfide into the sewage system requires frequently a significant capital investment for extensive sewer rehabilitation. The corrosion rate is about 5 mm/year in certain sections of the sewers in Japan [51] and Los Angeles County [52].

To date, several non-biological strategies have been developed for sulfide removal from wastewater, including chemical oxidation processes, which involve the use of air, ozone, hydrogen peroxide, stripping, and metal precipitation [47]. The use of these oxidizing agents results in chemical waste generation, high costs, and unwanted sulfate production. An alternative treatment process involves the biological formation of elemental sulfur, an insoluble intermediate that can be separated

from the liquid phase and reused [53]. Chemical oxidation involves a series of complex reactions and the formation of intermediates such as polysulfide, sulfur, thiosulfate, sulfite, and sulfate. The reactions can be catalysed by metal ions, and the reaction products depend on conditions such as pH and sulfide/oxygen ratio [54]. By contrast, biological sulfide oxidation can proceed under aerobic, anoxic, and even anaerobic conditions. Under these conditions, oxygen, nitrate, or carbon dioxide are used as electron acceptor, respectively [55–58]. Therefore, an aerobic post-treatment system based on autotrophic sulfide oxidizing bacteria could be envisaged to reduce the concentration of hydrogen sulfide in the outgoing wastewater converting it to elemental sulfur. Partial biological oxidation of sulfide is an inexpensive strategy, as S° is non-soluble and thus can be removed from the wastewater.

Our work is the first step towards the use of natural bacterial consortia for the treatment of real, sulfate-rich effluents from vegetable oil processing industries. Further efforts are still needed to improve reactor configuration in order to enable the treatment of larger wastewater batches in a reasonable period of time, as well as to ensure the robustness of the consortium against fluctuations in sulfate and nutrient concentrations in such wastewater effluents.

Acknowledgments

The authors are indebted to the technical staff of ALICORP S.A.A. for their kind collaboration during the sampling process. We also thank Kristie Tanner for language correction and critical reading of this manuscript.

Conflicts of Interest

The authors have no financial conflicts of interest to declare.

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